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Genetics

Transformation of *Paramecium* by microinjection of a cloned serotype gene

(ciliates/autonomous replication/surface antigen genes/macronucleus/gene expression)

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ABSTRACT **Paramecia of a given serotype express only one of several possible surface proteins called immobilization antigens (i-antigens). A 16-kilobase plasmid containing the gene for immobilization antigen A from *Paramecium tetraurelia*, stock 51, was injected into the macronucleus of deletion mutant d12, which lacks that gene. Approximately 40% of the injected cells acquired the ability to express serotype A at 34°C. Expression appeared to be regulated normally. The transformed cells, like wild type, could be switched to serotype B by antiserum treatment and culture at 19°C; on transfer to 34°C, they switched back to serotype A expression. Many of the lines retained the ability to express serotype A until autogamy, when the old macronucleus is replaced by a new one derived from the micronucleus. DNA from transformants contained the injected plasmid sequences, which were replicated within the paramecia. No evidence for integration was obtained. The majority of replicated plasmid DNA comigrated with a linearized form of the input plasmid. Nonetheless, the pattern of restriction fragments generated by transformant DNA and that generated by input plasmid DNA are identical and consistent with a circular rather than a linear map. These conflicting observations can be reconciled by assuming that a mixture of different linear fragments is present in the transformants, each derived from the circular plasmid by breakage at a different point. Copy-number determinations suggest the presence of 45,000–135,000 copies of the injected plasmid per transformed cell. These results suggest that the injected DNA contains information sufficient for both controlled expression and autonomous replication in *Paramecium*.**

Stable DNA-mediated genetic transformation may occur by either of two basic mechanisms: integration into host chromosomes or independent replication. Integration may come about by homologous recombination with genomic DNA and replacement of a section of the host chromosome with a portion of the introduced DNA. Integration may also occur at numerous sites in the genome by illegitimate recombination or by the introduction of sequences within elements such as Ti plasmids, retroviruses, or mobile genetic elements. Once integrated, replication is under chromosomal control. However, if the DNA is introduced as part of an autonomously replicating plasmid, it may be replicated independently of the chromosome under the control of plasmid sequences. In yeast it has been shown that transforming DNA can be replicated independently of both chromosomal and plasmid replication origins if the introduced DNA contains appropriate autonomously replicating sequences (ARS) (1). Replication may occur as circles, or, if appropriate, telomeric sequences are provided as linear molecules (2). However, little is known about the sequences necessary for independent replication, and virtually nothing is known about the control of the copy number of transformed DNA that is independently replicated.

There are no previous reports to our knowledge of transformation in *Paramecium* using cloned or isolated genomic materials, although transfers of nucleoplasm have been shown to change several different characteristics (3–5). It has been reported (6, 7) that *Stylonychia* can be transformed to G418 resistance. Tondravi and Yao (8) have demonstrated the transfer of free ribosomal DNA (rDNA; RNA-encoding DNA) by microinjection into the macronucleus of *Tetrahymena*. Production of transformants was favored because the injected rDNA enjoyed a selective advantage over the rDNA of the recipient.

A serotype of *Paramecium* is distinguished by the expression of one of several specific surface proteins called immobilization antigens (i-antigens) (9). In this paper we present evidence that a plasmid containing the gene for i-antigen A of *Paramecium tetraurelia* replicates within the macronucleus of a mutant strain that lacks that gene. In these transformed cells the gene for i-antigen A appears to be expressed under normal cellular control.

MATERIALS AND METHODS

Paramecia. Wild-type cells were *P. tetraurelia*, stock 51.s. The Mendelian mutant d12 was isolated after X-irradiation by J. Forney and L. Epstein (personal communication) as a line unable to express serotype A. The end of the macronuclear chromosome bearing the gene for i-antigen A was deleted beginning near the 5′ end of the gene. It also contained the twisty gene marker (10). Paramecia were cultured either in Cerophyl (Cerophyl, Kansas City, MO) or baked-lettuce medium. The methods of culture, induction of autogamy, harvesting cells, manipulation of serotypes, etc., have been described by Sonneborn (11, 12).

Cloned DNA. The cosmid α2 was isolated by Eric Meyer in our laboratory. In an effort to include telomeric sequences, stock 51 DNA was lightly digested with BAL-31 nuclease, and *Bam*HI linkers were added; after digestion with *Bam*HI, pBXDC6 cosmid arms, which had been cut at the *Bam*HI cloning site, were added. The clone was isolated by screening with known sequences of the isolated stock 51 i-antigen A gene (13); it includes the complete gene and flanking sequences.

pSA14SB consists of the 14-kilobase (kb) *Sal* I/*Bam*HI fragment of α2 subcloned into pT7/T3-18 (Bethesda Research Laboratories); it contains the i-antigen A gene and a portion of the flanking regions. A second plasmid derivative,

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Abbreviations: rDNA, RNA-encoding DNA; i-antigen, immobilization antigen; FIGE, field-inversion gel electrophoresis.

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pFA12BS, consists of the 12-kb *Bam*HI/*Sal* I fragment of α2 inserted into pT7/T3-18; the clone contains the region upstream of the i-antigen A gene. Restriction maps are given in Fig. 1.

Microinjection. Most of the microinjections were carried out by the method developed by Koizumi and described by Knowles (14–16) as modified for injection into the macronucleus (4). In some of the early injections, the technique described by Tondravi and Yao (8) was used. CsCl-purified DNA was suspended in the medium described by Tondravi and Yao (8). Prior to injection, cells were cultured in Cerophyl medium at maximum fission rate and had undergone autogamy a few fissions before the time of injection. Glass needles used in microinjection were 1–2 μm in diameter at their tips, and 5 or 6 pl of fluid were injected into the macronucleus. After injection, cells were transferred to Cerophyl or baked-lettuce medium and placed at 34°C, a temperature known to induce expression of serotype A in wild type (11). The next day they were fed with lettuce medium, which was more suitable for culture at high temperature. Cultures were maintained with an excess of fresh medium; under these conditions cells multiplied approximately four times per day. While the cultures were kept in logarithmic phase, autogamy was not observed for at least 30 or more fissions after injection and at intervals thereafter.

Appropriate control injections were performed. For example, calf thymus DNA and also pFA12BS, which contained only the upstream flanking sequences but not the i-antigen A gene, were injected. No transformants were obtained from these controls.

Preparation of DNA. Preparation of cosmid and plasmid DNAs was by the methods described by Maniatis *et al.* (17). *Paramecium* DNA was prepared as follows: lysates were made by resuspending 200,000 cells (containing ≈30 μg of DNA) in 0.2 ml of culture medium and adding this quickly to 0.4 ml of NDS medium (1% $NaDodSO_4$/0.5 M Na_2-EDTA/100 mM Tris·HCl, pH 9.5) at 65°C. After 48 hr at 65°C, the lysates were stored at 4°C, where they were stable for months. DNA was prepared from the lysates by three phenol extractions, dialysis against TE buffer (10 mM Tris·HCl/0.5 mM EDTA, pH 8.0), pervaporation at 4°C to approximately 0.5 ml, and precipitation with alcohol. Mitochondrial DNA was prepared as described by Suyama and Preer (18).

Electrophoresis. Standard methods were utilized, except for field-inversion gel electrophoresis (FIGE), which was used to resolve macronuclear genomic DNA (19). These runs were made in 0.8% Seakem HE agarose in 0.5× TBE buffer (1× TBE is 0.089 M Tris borate/0.089 M boric acid/2 mM EDTA, pH 8.0) at 2 V/cm, with cooling to 15°C and a switching cycle of 3 sec forward/1 sec reversed. Lysates with dye marker (17) containing sucrose (50% wt/vol) were carefully loaded into the wells by using pipettes with tips of ≈1-mm inside diameter. In trial runs markers were mixed with the lysing medium, and it was established that the positions of the bands at the end of the run were not affected appreciably by the lysing medium.

Southern Blot Hybridization. Hybridization was carried out under standard conditions (17). DNA was transferred to nitrocellulose and probed with nick-translated [^{32}P]dCTP-labeled pSA14SB DNA (specific activity, 1×10^8 cpm/μg). Hybridization was at 42°C in 50% formamide containing 5× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate), 1× Denhardt's solution (17), 50 mM Hepes (pH 7.0), and 1 mM EDTA. Filters were washed twice with 2× NaCl/Cit containing 0.1% $NaDodSO_4$ at 68°C for 30 min and then once in 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$ for 30 min at 68°C prior to autoradiography. Slot-blotting was carried out by using a Schleicher & Schuell Minifold under conditions recommended by the manufacturer.

RESULTS

Transformation Is Induced by Cosmid α2. Initial microinjection experiments were carried out with a 36-kb cosmid designated α2. This cosmid includes the 8.4-kb transcription unit of the i-antigen A gene, 13 kb of upstream sequence, and 4 kb of downstream sequence. Cells of deletion strain d12 were injected with α2 and then isolated and allowed to produce clones under A serotype-inducing conditions (34°C). Of 212 clones, 7 had transformed to A-expressing clones when tested 5–8 fissions after injection. In all of the clones but 1, 100% of the cells expressed serotype A. In the exceptional clone, 80% of the cells expressed A, and the remainder expressed other serotypes. Four of the 7 clones stably expressed serotype A 30–35 fissions after injection. Three of the 7 clones, including the clone that was originally 80% A-expressing, were unstable; the percentage of A-expressing cells decreased in each of these unstable clones with successive fissions. Autogamy normally occurs in these lines 30–40 fissions after injection, at which time the parental macronucleus in each cell is replaced by a new one derived from the micronucleus. All transformant lines lost the ability to express serotype A within 4–5 fissions after autogamy. This observation is consistent with the expected fate of DNA introduced into the macronucleus.

Paramecia can alter their surface antigens under different environmental conditions. Wild-type cells of stock 51 that express the A serotype can be induced to switch to non-A-expressing types. For example, treatment of stock 51 sero-

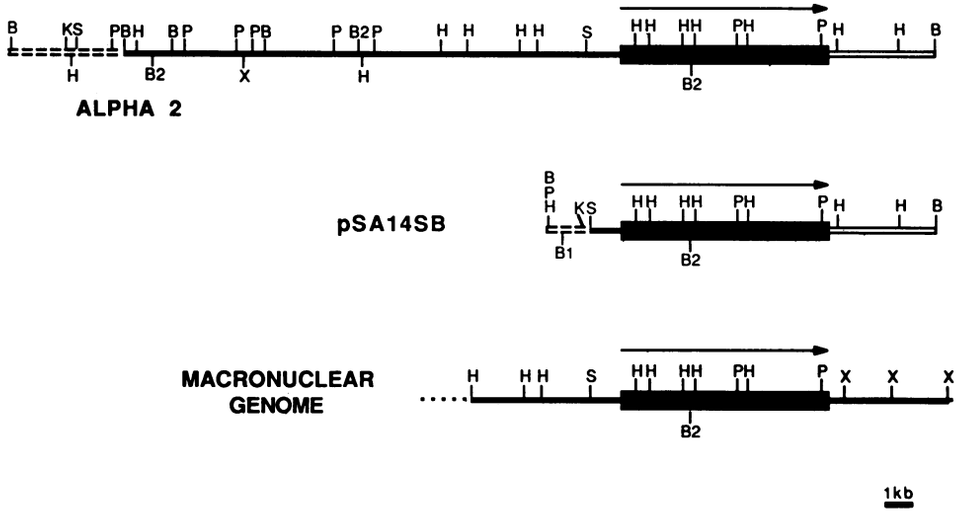

FIG. 1. Restriction maps of the i-antigen A gene and flanking sequences in the cosmid α2, the plasmid pSA14SB, and a portion of the macronuclear genome. The circular cosmid and plasmid are represented linearly. The thick line and arrow indicate the transcribed portion of the i-antigen A gene. The dashed lines indicate the vector sequences. The open and solid lines downstream of i-antigen A gene in α2 and the macronuclear genomic copies emphasize that these sequences are different. B, *Bam*HI; B1, *Bgl* I; B2, *Bgl* II; H, *Hin*dIII; K, *Kpn* I; P, *Pst* I; S, *Sal* I; X, *Xho* I.

type A cells with anti-A antiserum followed by removal of the antiserum and culture of the cells at 19°C causes the cells to become serotype B (20). If these paramecia are subsequently returned to 34°C, they stop expressing serotype B and resume expression of serotype A. To determine whether A expression in d12 transformants exhibited such control, transformants were treated in a similar manner. Serotype A-expressing cells from a clone that had been transformed with α2 were exposed to homologous serum and placed at 19°C. After approximately seven fissions, all of the resulting subclones consisted of cells that were 100% B. The subclones were then placed at 34°C, where they underwent four fissions per day. After 2 days, all cultures consisted of 100% A-expressing cells.

One aspect of the structure of α2 is noteworthy. No less than 6 kb of the upstream sequences in α2 correspond to the map of the region of the macronuclear genome bearing the i-antigen A gene (ref. 13; J. Forney, personal communication; Fig. 1). However, the sequences immediately downstream of this gene in α2 are different from those in the macronuclear genomic map. The origin of the downstream sequences will be discussed later. The precise point of divergence between the map of α2 and the map of the macronuclear genome is not known but it must lie in an ≈1-kb region between the distal *Pst* I site in the i-antigen A gene and the nearest *Xho* I site in the genomic map.

High-Frequency Transformation Is Induced by pSA14SB. To facilitate molecular analysis of the injected DNA, a region containing the i-antigen A gene and flanking sequences was subcloned from α2 to generate a 16-kb plasmid designated pSA14SB. This plasmid contains the 14-kb *Sal* I–*Bam*HI fragment of α2 cloned into the expression vector pT7/T3-18. pSA14SB contains the i-antigen A gene with about 1.5 kb upstream and 4 kb downstream (Fig. 1). The region downstream of this gene in pSA14SB is identical to that in α2. Using the i-antigen A gene deletion strain d12 as recipient, 41% (50/121) of the cells injected with pSA14SB generated populations that expressed serotype A 10 fissions after injection. All of the cells in most of these populations were serotype A. Thirty-five of the 50 A-expressing clones were retested after eight more fissions. Of these, approximately half (18/35) had lost the ability to express A. However, the remaining clones (21% of those originally injected) expressed serotype A stably; loss of serotype A after 18 fissions was rare except at autogamy. As in the case of the transformants induced with α2, transformants originating from pSA14SB showed normal expression when placed under environmental conditions favoring either serotype A or B and lost serotype A at autogamy.

Transformation was strongly dependent upon the amount of DNA injected and the site of injection. While the standard injection of 5 pl of pSA14SB DNA at 5 μg/μl (≈10^6 molecules) generated 41% (50/121) A gene-containing clones 10 fissions after injection, 1 μg/μl gave 5% (1/20) and 0.1 μg/μl produced 0% (0/21). Injection into the macronucleus was required for transformation. No transformants (0/50) were obtained when 10 pl of DNA (twice the standard amount injected into the macronucleus) was injected into the cytoplasm.

Injected Sequences Are Not Integrated. To investigate the state of the injected DNA, transformant DNA was analyzed by FIGE (ref. 19). Cells were lysed under conditions that minimized breakage of DNA. The procedure permitted clear separation of the macronuclear chromosomes, estimated to be ≈300 kb (21), from smaller DNA of the size of pSA14SB, 16 kb. Fig. 2*A* shows the ethidium bromide staining pattern of *Paramecium* DNA separated by FIGE. The bulk of the *Paramecium* DNA migrated as a broad smear, presumably reflecting heterogeneity in the size of macronuclear chromosomes. A faster migrating discrete band (labeled "m") is also apparent in each of the ethidium bromide-stained lysates.

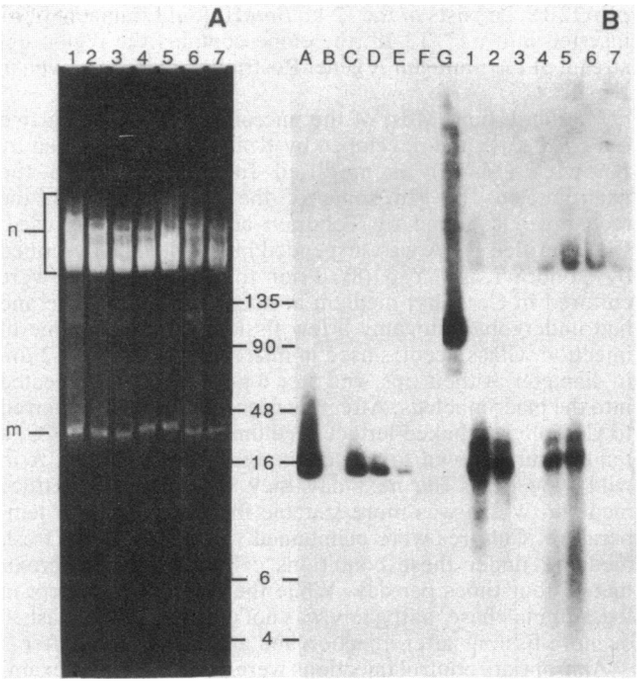

FIG. 2. (*A*) Agarose FIGE of *Paramecium* DNA. Samples of lysed paramecia (20 μl per sample, each containing ≈1 μg of DNA) were electrophoresed on a 0.8% agarose gel as described. The gel was stained with ethidium bromide and photographed. Lanes: 1–5, microinjected transformant DNAs; 6, parental stock 51 DNA; 7, mutant d12 DNA. The broad band "n" represents the bulk of the macronuclear chromosomal DNA. The lower band "m" is mitochondrial DNA. (*B*) DNA blot analysis of transformant lines. DNA in the agarose gel shown in *A* was transferred to nitrocellulose and probed with labeled pSA14SB DNA. Lanes: A–F, Kpn I-linearized pSA14SB DNA at 12 ng (lane A), zero (lane B), 2.6 ng (lane C), 0.7 ng (lane D), 0.15 ng (lane E), and 0.03 ng (lane F); G, 10 ng of pSA14SB uncut; 1–5, transformants T17.9A (lane 1), T17.9B (lane 2), T17.6 (lane 3), T19.1 (lane 4), and T19.2 (lane 5); 6, wild-type 51; 7, strain d12. The autoradiogram was exposed for 18 hr.

This band migrates with a mobility of about 40 kb and comigrates with DNA isolated from purified *Paramecium* mitochondria (results not shown). *Paramecium* mitochondrial DNA is known to be a 41-kb linear DNA species (22).

The DNA shown in Fig. 2*A* was transferred to nitrocellulose and hybridized with ^{32}P-labeled pSA14SB DNA (Fig. 2*B*). Noninjected control lysates from the recipient cell line, d12 (Fig. 2*B*, lane 7), and transformant lysates (Fig. 2*B*, lanes 1–5) showed faint hybridization in a region of the gel corresponding to the mobility of intact macronuclear chromosomes. This result was expected because sequences upstream of the i-antigen A gene are not deleted in d12 and are present in the pSA14SB probe. The intensity of hybridization in this region was approximately identical in transformant and control lysates. Compare the d12 lysate (Fig. 2*B*, lane 7) with the T17.9A lysate (Fig. 2*B*, lane 1). Lysates from transformants showed two discrete bands of higher mobility that hybridized with the probe. The molecular form of the upper hybridizing band is unknown; the lower more prominent band migrated with a mobility similar to that of linear pSA14SB (in Fig. 2*B*, compare lanes 1, 2, 4, and 5 with lanes A, C, D, and E). No hybridization was detected with noninjected control lysates of d12 at this position (Fig. 2*B*, lane 7). In each of the transformant lines except T17.6 (lane 3), 100% of the cells were expressing i-antigen A when the lysates were prepared. Although T17.6 showed 100% i-antigen A expression 10 fissions after microinjection, it showed 40% expression after 14 fissions, and no expression

after 18 fissions or after 22 fissions, when DNA was prepared. Thus, loss of i-antigen A expression correlates with lack of hybridization in the 16- to 26-kb region.

Injected Sequences Are Replicated. Lane 1 (T17.9A) and Lane 2 (T17.9B) of Fig. 2*B* show lysates of a clone derived from a single transformant taken 20 and 30 fissions after injection, respectively. It is clear from inspection of these lanes that the plasmid sequences were being replicated, for if they were not, the 10 fissions occurring between 20 and 30 fissions would have diluted the signal by a factor of 2^{-10} or 1/1024, whereas the intensities of the bands are similar. The conclusion that the sequences were replicated is also apparent from consideration of the distribution of the $\approx 10^6$ molecules of the plasmid injected into each host cell. Thirty fissions after injection, these molecules would have to be distributed among 2^{30} (10^9) cells—i.e., one molecule for every 1000 cells. This prediction is clearly at variance with the fact that 100% of the cells expressed serotype A in many transformed clones at 30 fissions.

Transformant DNA Comigrates with Linearized pSA14SB but Maps Like Circular pSA14SB. Although there was considerable diversity in size of the replicating i-antigen A gene-containing DNA in the transformants, most of the hybridizing material migrated to the same position as pSA14SB, which had been linearized with *Kpn* I. Moreover, the uncut open circular and supercoiled forms of pSA14SB migrated slowly in these gels (Fig. 2*B*, lane G). These results suggest strongly that most of the replicating DNA was converted to linear form. To test this idea, we attempted to define the termini of these molecules. If the cleavage to generate the linear form was highly sequence-specific in *Paramecium*, cleavage with a restriction enzyme that cuts once in pSA14SB should generate two distinct DNA fragments. On the other hand, nonspecific cutting would generate a series of different ends relative to a fixed site and generate a smear of fragments. Purified DNA from transformant lysates was cut with different enzymes, and the fragment pattern was compared to that from pSA14SB DNA. Labeled pSA14SB DNA was the probe in blot hybridizations. The results are shown in Fig. 3. When transformant DNA was cleaved with the single-cut enzymes *Kpn* I and *Bgl* I, a smear of hybridizing DNA is apparent (lanes 3, 9, and 15 and lanes 4, 10, and 16, respectively). The same result was obtained with *Bgl* II, which also cleaves once (data not shown). When cut with enzymes that make multiple fragments, such as *Hin*dIII or *Kpn* I/*Pst* I, there was little apparent difference in the hybridization pattern of transformant DNA and pSA14SB DNA (lanes 2, 8, and 14 and lanes 5, 11, and 17, respectively). From these results it is clear that all plasmid sequences were present in the transformants. Restriction maps constructed from the latter data yielded a circular restriction map for transformant DNA. Taken together with the results described earlier, these restriction data are not consistent with a specific-cleavage model to generate the linear form and indicate that the transformant DNA consists of a collection of linear molecules that have been generated by cleavage of the injected circular DNA at different points.

Copy Number. It is difficult to determine the copy number of the injected DNA in transformants from the data in Fig. 2 because of the potential varying efficiency of transfer from the gel to nitrocellulose. To assess quantitatively pSA14SB copy number, slot-blot-hybridization experiments were carried out (Fig. 4). Purified total DNA preparations from four separate transformant lines, d12, or strain 51 were spotted on filters and probed with nick-translated pSA14SB DNA. Known amounts of purified pSA14SB DNA were also fixed to the filter. This analysis revealed that transformant DNA copy number (expressed as the number of monomeric pSA14SB equivalents) was high, ranging from 45,000 (T13.5) to 135,000 (T19.1) copies per cell. Transformant DNA comprises between 0.5% and 1.6% of total-cell DNA in each of the transformant lines. The level of ploidy in the macronucleus of *Paramecium* has been estimated to be about 1700 (23). Therefore, transformant lines maintain about 25–80 times the number of i-antigen A gene copies present in the wild-type stock 51 macronucleus.

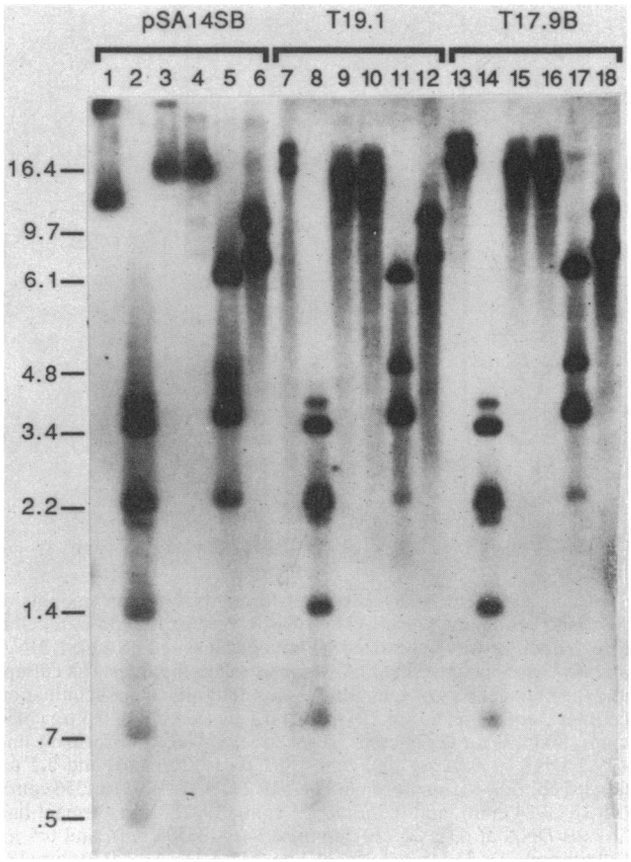

FIG. 3. Restriction fragment analysis of transformants. Restriction fragments of DNA from pSA14SB and two transformants (T19.1 and T17.9B) were electrophoresed on 1% agarose, transferred to nitrocellulose, and probed with pSA14SB. Lanes: 1, 7, and 13, uncut pSA14SB, T19.1, and T17.9B, respectively; 2, 8, and 14, cut with *Hin*dIII; 3, 9, and 15, cut with *Kpn* I; 4, 10, and 16, cut with *Bgl* I; 5, 11, and 17, cut with *Pst* I/*Kpn* I; 6, 12, and 18, cut with *Bgl* II/*Kpn* I. The autoradiogram was exposed for 18 hr.

DISCUSSION

We have shown that transformation can be obtained with high frequency in *Paramecium* by microinjecting macronuclei with plasmids containing the i-antigen A gene and flanking sequences. This DNA is replicated, maintained in high copy number, and finally lost when the transformed macronucleus is destroyed at autogamy. The gene for i-antigen A appears to be expressed in a normal fashion in transformants: it is subject to the regulatory mechanisms that enforce mutual exclusion of other antigens and govern response to changes in temperature.

Injected sequences appear not to be integrated into the macronuclear chromosomes in transformants, since the bulk of the injected sequences do not comigrate with chromosomal DNA. Moreover, restriction enzyme analysis reveals no evidence of fragments whose size has been altered by integration into macronuclear DNA. Nevertheless, we cannot rule out the possibility that a small fraction (<5%) of the copies is integrated.

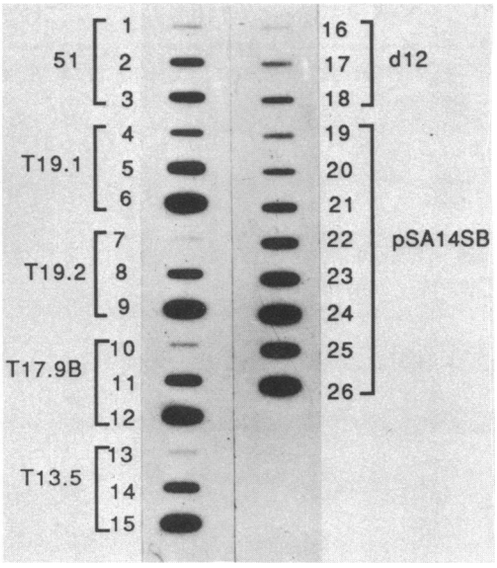

FIG. 4. Slot-blot analysis of transformants. Various amounts of purified *Paramecium* or pSA14SB DNA were fixed onto nitrocellulose. The filter was hybridized with nick-translated pSA14SB DNA and processed as described. After processing, the filter was cut up, and the radioactivity of each sample was determined in a scintillation counter. Slots: 1–3, strain 51 DNA at 0.1 μg (335 cpm), 0.5 μg (1450 cpm), and 1.0 μg (2278 cpm), respectively; 4–6, transformant line T19.1 DNA at 0.01 μg (995 cpm), 0.1 μg (4120 cpm), and 0.5 μg (10,867 cpm); 7–9, transformant line T19.2 DNA at 0.01 μg (356 cpm), 0.1 μg (1924 cpm), and 0.5 μg (9532 cpm); 10–12, transformant line T17.9B DNA at 0.01 μg (307 cpm), 0.1 μg (2650 cpm), and 0.5 μg (7496 cpm); 13–15, transformant line T13.5 DNA at 0.01 μg (182 cpm), 0.1 μg (1790 cpm), and 0.5 μg (6830 cpm); 16–18, d12 DNA at 0.5 μg (258 cpm), 1.0 μg (407 cpm), and 2.0 μg (646 cpm); 19–24, pSA14SB DNA at 0.075 ng (398 cpm), 0.15 ng (633 cpm), 0.38 ng (1454 cpm), 0.75 ng (2430 cpm), 1.5 ng (4217 cpm), and 3.75 ng (8910 cpm); 25, 1.5 ng of pSA14SB DNA and 0.5 μg of d12 DNA (4769 cpm); 26, 3.75 ng of pSA14SB DNA and 0.5 μg of d12 DNA (8666 cpm). The autoradiogram was exposed for 2 hr. *P. tetraurelia* contains about 150 pg of DNA per cell (J.R.P., unpublished data).

FIGE gel analysis revealed that transformants contain two distinct electrophoretic species of DNA that hybridize with input plasmid DNA. One species migrates at about 26 kb, and the other migrates at 16 kb. The smaller of these forms has mobility similar to linearized pSA14SB. The molecular form of the other band is unknown. It could be an alternate conformation of the lower form, a dimeric form, or a replicative intermediate. Further experiments are necessary to understand the relationship between the two bands.

Restriction enzyme analysis shows that all sequences present in pSA14SB are present in the transformants. However, there are two puzzling facts about the restriction digestion products of transformant DNA. First, if the DNA in transformants is linear, enzymes that cut supercoiled pSA14SB DNA once would be expected to yield two distinct fragments when transformant DNA is cut. Instead a broad smear results. Second, the pattern of fragments generated by enzymes that multiply cut pSA14SB is identical to that shown with purified pSA14SB and is consistent with a circular restriction map. The simplest model to explain these observations is that the injected supercoiled DNA becomes linearized by cuts at different points, and subsequently some fraction of this DNA is capable of autonomous replication. The resulting molecules represent a heterogeneous collection, many of which have different ends. It is clear, however, that this collection does not represent a random sample of all possible permutations of linearized molecules. If the cleavage sites were random, treatment with single-cut enzymes—e.g., *Kpn* I or *Bgl* I—would be expected to generate molecules with an average size of about 8 kb. The data shown in Fig. 3 indicate that the average molecular weight after such treatment is significantly greater than 8 kb. This apparent nonrandom cleavage could be a consequence of favored sites for cleavage or random cleavage followed by selection for molecules capable of replication. We cannot rigorously exclude other more complicated explanations—e.g., that the nonintegrated DNA results from high-level replication of a small number of integrated copies or that the transformant molecules, while having the mobility of 16-kb linear molecules, are circular with an unusual topological structure. Thus, the hypothesis that the transformant DNA consists of a mixed population of linear molecules derived from cleavage of pSA14SB at different points must be confirmed with more direct evidence before one can be certain about the physical state of the transformed DNA.

Further work will be required to determine what sequences are necessary for replication. The fact that large numbers of molecules must be injected (about 10^6) to obtain high-level transformation frequency may reflect the fact that only a small number of the injected molecules actually replicate initially or are modified to become capable of replication.

It is noteworthy that the downstream region flanking the i-antigen A gene in the cosmid α2 and pSA14SB is totally different from the sequence known to be downstream from the A gene derived from clones from other libraries of macronuclear DNA (13). Conceivably sequences downstream of the i-antigen A gene in the macronucleus are heterogeneous, and α2 may have been derived from a macronuclear copy that differs from the type previously described (Fig. 1). Rearrangements that occur during processing are well-documented in other ciliates (24, 25) and recently have been described downstream of the i-antigen A gene in *Paramecium* (J. Forney and E. Blackburn, personal communication).

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